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Cellular Basis for ADT-Induced Acceleration of Sarcopenia

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14. ABSTRACT Currently the only proposed treatment for ADT-induced muscle wasting is exercise based interventions. However, the intensity of exercise required coupled with the susceptibility of prostate cancer patients to fractures due to 1) the already high incidence of bone metastasis and 2) ADT-induced acceleration in osteoporosis emphasizes the need for other therapies. In order to devise effective therapies, an understanding as to how ADT results in severe and debilitating loss in skeletal muscle and overall performance is required. The goal of this proposal is to employ pre-clinical models to gain insight into the cellular and molecular mechanisms responsible for ADT-induced loss of skeletal muscle regenerative potential. Specifically, the aims are designed to address how ADT affects the maintenance and function of Pax7 expressing resident stem cells of skeletal muscle, satellite cells (SCs), which are absolutely required for optimal skeletal muscle growth and regeneration. The completion of the aims outlined in this proposal should provide insights into the extent to which abnormalities in stem cell function and thereby regenerative capability contribute to the acceleration of sarcopenia observed in prostate cancer patients undergoing ADT. In addition, this proposal intends to employ pre-clinical genetic strategies to determine the utility of stimulating the expression of genes known to maintain SC number and function for the ability to resist ADT-induced loss of skeletal muscle regeneration. Ultimately, the elucidation of regenerative strategies to circumvent androgen supplementation to sustain skeletal muscle should be of tremendous benefit towards research that strives to improve the quality of life and well-being of prostate cancer patients receiving ADT.					
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1. Introduction:

Currently the only proposed treatment for ADT-induced muscle wasting is exercise based interventions¹⁻³. However, the intensity of exercise required coupled with the susceptibility of prostate cancer patients to fractures due to 1) the already high incidence of bone metastasis and 2) ADT-induced acceleration in osteoporosis emphasizes the need for other therapies. In order to devise effective therapies, an understanding as to how ADT results in severe and debilitating loss in skeletal muscle and overall performance is required. The goal of this proposal is to employ pre-clinical models to gain insight into the cellular and molecular mechanisms responsible for ADT-induced loss of skeletal muscle regenerative potential. Specifically, the aims are designed to address how ADT affects the maintenance and function of Pax7 expressing resident stem cells of skeletal muscle, satellite cells (SCs), which are absolutely required for optimal skeletal muscle growth and regeneration⁴. The completion of the aims outlined in this proposal should provide insights into the extent to which abnormalities in stem cell function and thereby regenerative capability contribute to the acceleration of sarcopenia observed in prostate cancer patients undergoing ADT. In addition, this proposal intends to employ pre-clinical genetic strategies to determine the utility of stimulating the expression of genes known to maintain SC number and function for the ability to resist ADT-induced loss of skeletal muscle regeneration. Ultimately, the elucidation of regenerative strategies to circumvent androgen supplementation to sustain skeletal muscle should be of tremendous benefit towards research that strives to improve the quality of life and well-being of prostate cancer patients receiving ADT.

2. Keywords:

Androgen deprivation, castration, skeletal muscle, regeneration, stem cells, satellite cells

3. Accomplishments:

Major goals of the project:

Aim 1 was to determine if androgen deprivation is sufficient to cause disruptions in SC quiescence.

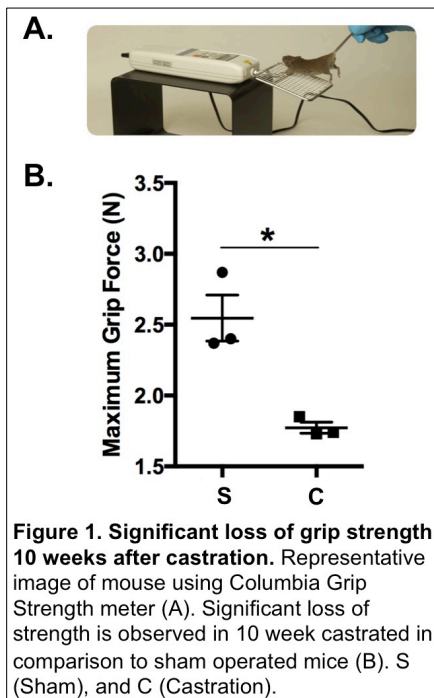
Aim 2 was to determine if androgens are regulators of SC fate to sustain the regenerative capacity of skeletal muscle.

Aim 3 was to determine if SC-specific overexpression of sprouty1 (Spry1) is sufficient to preserve SC pool size, function and skeletal muscle integrity in the absence of androgens.

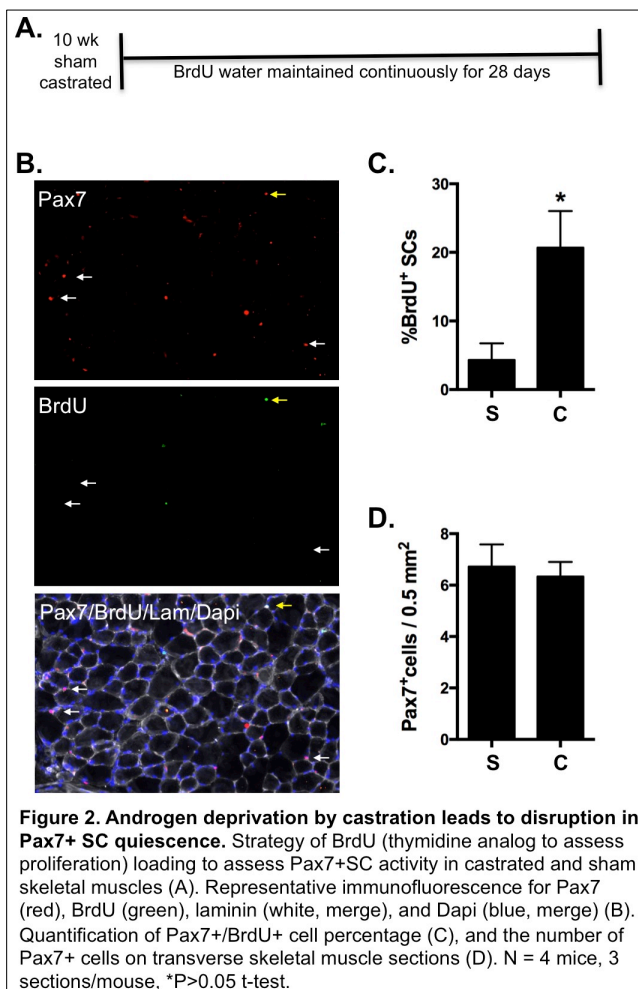
Accomplished goals:

Aim 1: Determination of quiescent and activated SCs from muscle cryosections.

Methods: To assess the ability of SCs to sustain quiescence in an androgen-deprived environment, sham operated and castrated mice were fed 0.5mg/ml BrdU (Sigma-aldrich) in drinking water supplemented with 5% sucrose for 10 weeks. This time frame correlates with significant losses in skeletal muscle performance^{5,6}. Strength was measured with a Columbia Grip Strength Meter. Subsequently, tibialis anterior (TA) muscles were collected, crysectioned and processed for sodium citrate antigen retrieval⁷. Processed skeletal muscle sections were then prepared for BrdU (abcam), laminin (sigma-aldrich, muscle fiber periphery) and Pax7 (DSHB) immunofluorescence. The numbers of BrdU+ Pax7 cells were counted to determine the extent of disruptions in SC quiescence and number. To assess SC molecular character after castration pure populations of SCs were isolated from lower-limb and forelimb muscles as described previously⁷. To FACs purify SCs we employed previously described forward and side-scatter profiles, negative selection for CD31/45 (BD Biosciences) and positive selection for α 7-integrin (ITGA7)⁷. This sorting strategy has been shown to enrich for Pax7 positive cells⁷. FACs purified SCs were sorted directly into Trizol LS (Invitrogen). Subsequently total RNA was column purified and DNase treated (Qiagen). To assess differences in the expression of key SC fate regulators including Hes1 and HeyL (Notch signaling mediators)^{8,9} and Spry1 (negative



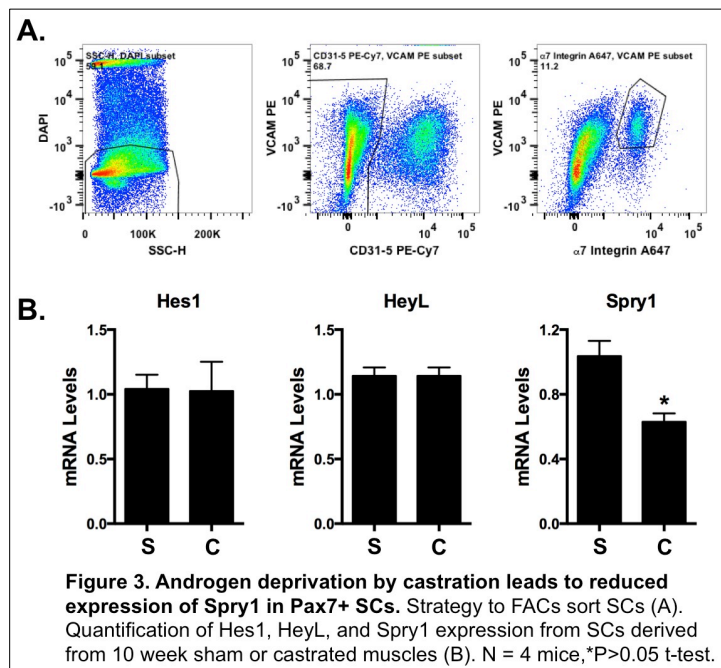
feedback regulator of RTK/FGF signaling)⁷, first-strand cDNA synthesis and subsequent qPCR was performed (Invitrogen; SuperScript First-Strand Synthesis and Platinum SYBR Green qPCR SuperMix-UDG with ROX). Reverse transcribed cDNAs were PCR amplified using a StepOnePlus real time PCR system (Applied Biosystems) in triplicate with validated primer sets obtained from Massachusetts General Hospital primer bank or Origene technologies. After amplification, the comparative Ct method was used to obtain a relative fold change in gene expression



for a specific primer set between SCs from sham and castrated mice standardized to the reference gene GAPDH⁷.

Results: Consistent with previous reports a significant decline in grip strength was observed 10 weeks after castration (Fig 1)^{5,6,10,11}. A significant increase in the proportion of Pax7 expressing SCs that were positive for BrdU, a thymidine analog that is used to trace dividing cells, was observed 10 weeks after castration (Fig 2). This suggests that a population of SCs have broken quiescence, a reversible state of growth arrest, in response to castration. Prolonged disruptions in quiescence can be associated

with eventual depletion of stem cell pools; therefore we enumerated Pax7+ SC number 10 weeks after castration. Unlike other modulations known to disrupt Pax7+ SC quiescence no



significant decline in Pax7+ SC pool size at homeostasis occurs in response to castration (Fig 2)^{8,12,13}. To assess intrinsic functional capacity we will perform clonal growth assays of SCs purified from skeletal muscles of castrated mice in defined conditions⁷. Examination of FACs purified SCs for the expression of genes that promote the maintenance of SCs during

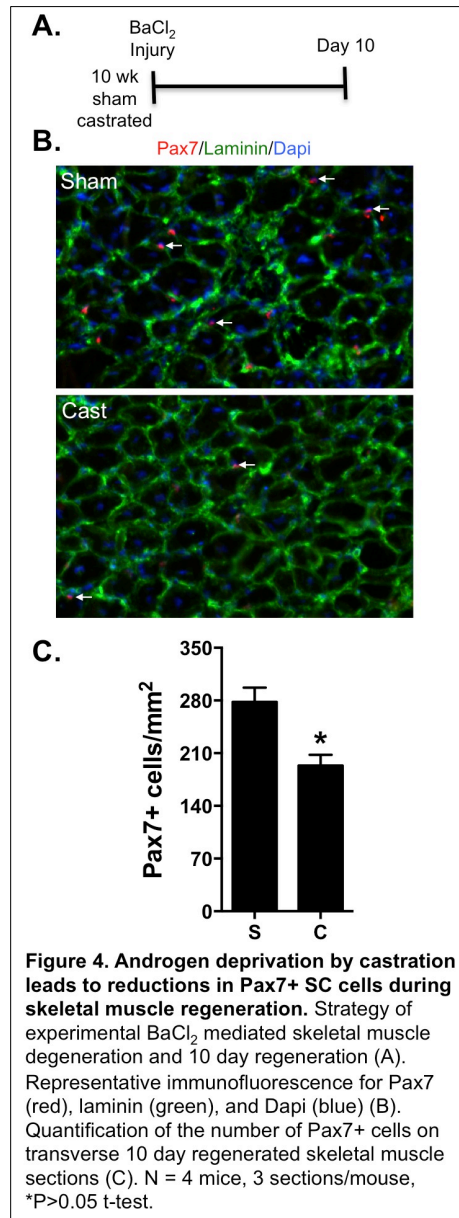
regeneration revealed reduced expression of the receptor tyrosine kinase/FGF feedback regulators sprouty1 (Spry1) as opposed to Notch effectors (Fig 3)⁷⁻⁹. As described in Aim 3 we have devised a strategy to overexpress Spry1 specifically in Pax7+ SCs as a means to prevent SC loss in androgen deprived regenerating skeletal muscles.

Statement of work progress: **Major Task 1.** Assessment of SC quiescence in response to castration (orchidectomy): Subtask 1. In-vivo BrdU loading following castration, we have completed these experiments and the results are depicted in Fig 2A-C. These data indicate that castration leads to disruption of quiescence, and activation of a higher proportion of SCs in the absence of a major direct muscle regenerative stimulus. Subtask 2. Characterization of SC fate following castration, we have completed these experiments and the results are depicted in Fig 2D. These data indicate despite disruption of quiescence no significant decline in Pax7+ SC number occurs following castration. **Major Task 2.** Assessment of SC molecular character and function following castration: Subtask 1. Assessment of SC molecular character following castration, we have conducted these experiments and the results are depicted in Fig 3. These data indicate reduced expression of Spry1 is a feature of SCs isolated from androgen deprived skeletal muscles, Subtask 2. Assessment of SC function in culture following castration, we intend to perform clonal culture experiments to determine whether SCs obtained from an

androgen-deprived environment possess intrinsic functional deficits that manifest in defined culture conditions.

Aim 2: Determination SC fate during regeneration.

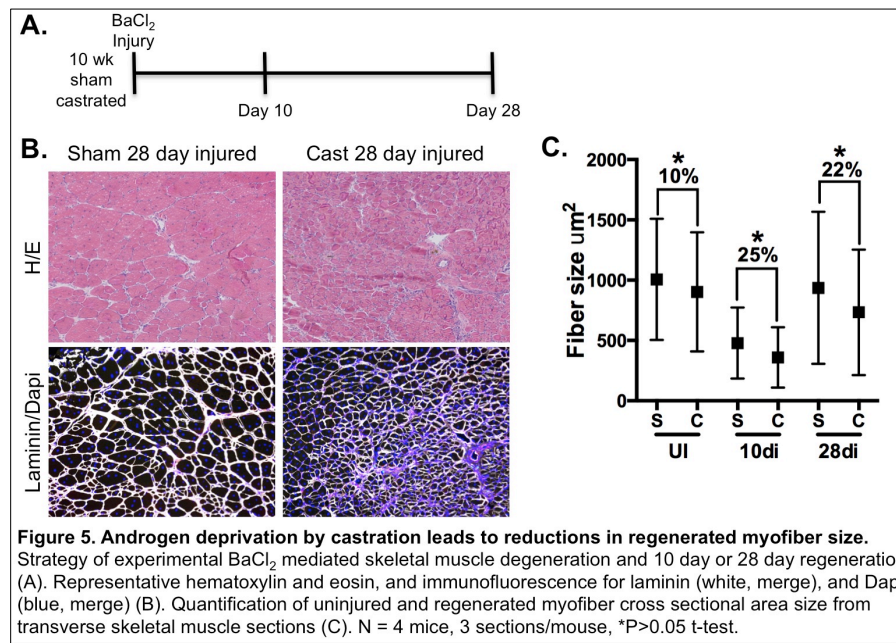
Methods: To assess SC fate during skeletal muscle regeneration TA muscles of castrated and sham mice were injured with 1.2% BaCl₂, a solution known to cause efficient



muscle fiber degeneration without affecting supporting cells or SC survival¹⁴. After recovery from injury mice were sacrificed and TAs processed for hematoxylin and eosin histology, and Pax7 and laminin (skeletal muscle fiber periphery) immunofluorescence. Skeletal muscle fiber size, and the size of the SC pool were determined.

Results: Analysis of Pax7+ SC immunofluorescence revealed a significant decline in SC number in 10 day regenerated skeletal muscles from mice that had been castrated 10 weeks prior (Fig 4). The decline in SC number was accompanied by a significant deficit in regenerated skeletal muscle morphology based on hematoxylin and eosin histology, and myofiber size based on laminin immunofluorescence in 10 and 28 day regenerated skeletal muscles from mice castrated 10 weeks prior (Fig 5). We intend to examine the mechanisms responsible for loss of SCs during regeneration in an androgen-deprived environment. Possible mechanisms

that could reduce Pax7+ SC numbers during regeneration in an androgen-deprived environment include insufficient activation, precocious differentiation, and apoptosis^{15,16}. Moreover, an androgen-deprived environment could manifest in intrinsic modifications in SCs that hinder



muscles of castrated mice.

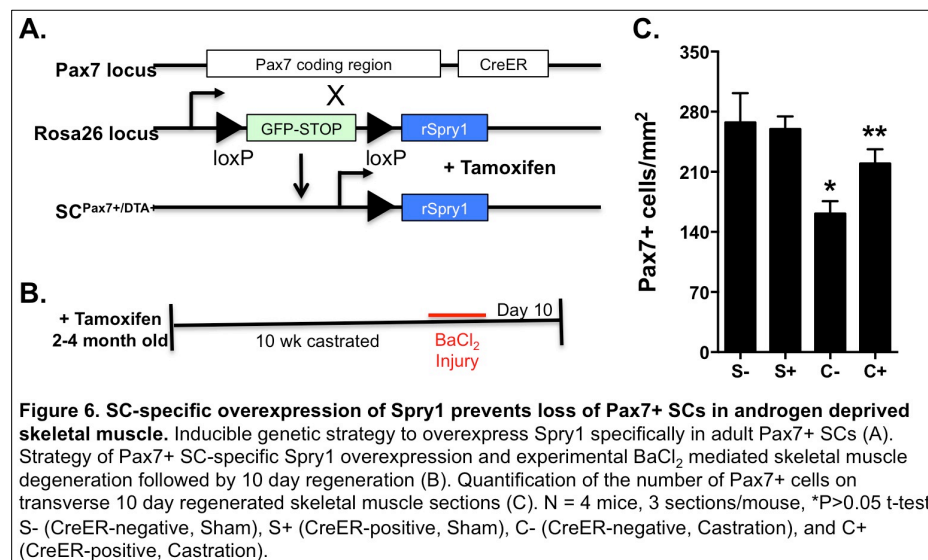
Statement of work progress: **Major Task 1.** Assessment of the ability of SC to enter cycle following castration and during skeletal muscle regeneration: Subtask 1. In-vivo BrdU IP injections following 10 weeks of castration and during skeletal muscle regeneration. We intend to perform these experiments within the upcoming year. Subtask 2. Assessment of SC fate and renewal following 10 weeks of castration and during skeletal muscle regeneration, we have completed some of these experiments as depicted in Fig. 4. These data indicate a significant decline in Pax7+ SCs and myogenic progenitors in 10 day regenerated castrated muscles, in the upcoming year we intend to obtain a more complete description of SC fate and renewal with additional markers of myogenesis. **Major Task 2.** Assessment of the regenerative capability of skeletal muscle in response to compound injuries following 10 weeks of castration: Subtask 1. Assessment of SC fate and renewal in response to primary and secondary injuries following 10 weeks of castration, we have conducted primary injuries as depicted in Fig 5. These data indicate some regenerative failure is evident in 10 day, and 28 day (primary) regenerated castrated skeletal muscles. In the upcoming year we intend to characterize SC fate and renewal after primary and secondary/compound (28 day + 28 day) regenerated castrated and sham skeletal muscles.

function during regeneration^{7,15-19}. We intend to perform FACS-based and immunofluorescence analysis with various markers for SC fate, inflammation, fibrosis and apoptosis in regenerating skeletal

Aim 3: Determination of SC fate in WT and Spry1OX transgenic mice during muscle regeneration.

Methods: To determine the consequences of SC-specific Spry1 overexpression in androgen deprived regenerating skeletal muscle we have generated Spry1OX transgenic mice. Spry1OX mice enable SC specific overexpression of Spry1 after administration of tamoxifen (TM)⁷. All mice used were heterozygote for the CAG^{rSpry1} transgene and administered TM, which does not influence wild type function⁷. For all experiments controls were negative for CreER. The TA muscles of castrated and sham operated control and Spry1OX mice were injured with 1.2% BaCl₂ as described in Task2. To assess Pax7+ SC number during skeletal muscle regeneration 10 days after recovery from injury mice were sacrificed and TAs processed for Pax7, and laminin immunofluorescence.

Results: Assessment of Pax7+ SC numbers reveal that Spry1 overexpression specifically in SCs is sufficient to attenuate loss of SCs in regenerating androgen deprived



skeletal muscles (Fig 6). We intend to examine whether Spry1 overexpression prevents SC loss by promoting proliferation, preventing apoptosis and/or differentiation

in an androgen deprived environment. In parallel we will also examine how SC specific Spry1 over expression affects regenerated skeletal muscle morphology and size.

Statement of work progress: **Major Task 1.** Assessment of skeletal muscle integrity, strength and SC quiescence in response to castration: **Subtask 1.** In-vivo BrdU loading following castration of Pax7CreER; Spry1OX mice; **Subtask 2.** Characterization of SC fate following

castration of Pax7CreER; Spry1OX mice; and Subtask 3. Assess skeletal muscle strength and integrity. **Major Task 2**. Assessment of SC molecular character and function following castration: Subtask 1. Assessment of SC molecular character and function following castration of Pax7CreER; Spry1OX mice. **Major Task 3**. Assessment of the ability of SCs to enter cycle following castration and during skeletal muscle regeneration: Subtask 1. Assessment of SC fate and renewal following 10 weeks of castration and during skeletal muscle regeneration of Pax7CreER; Spry1OX mice. **Major Task 4**. Assessment of the regenerative capability of skeletal muscle in response to compound injuries following 10 weeks of castration: Subtask 1. Assessment of SC fate and renewal in response to primary and secondary injuries following 10 weeks of castration of Pax7CreER; Spry1OX mice. We have successfully generated the Pax7CreER; Spry1OX mice. Initial experiments indicate castration induced loss of Pax7+ SCs in 10 day regenerated skeletal muscles is attenuated upon specific over expression of Spry1 in SCs. Over the next 2 years we anticipate completing the above tasks with the Pax7CreER; Spry1OX mice.

Opportunities for training and professional development:

Dr. Chakkalakal was an invited speaker at the Rochester Aging Research Center symposium day. The presentation focused on the work described in this annual review.

Results disseminated to communities of interest:

Nothing to report.

Plan for next reporting period to accomplish goals:

The present studies have provided insight into some of the cellular and molecular features of ADT-induced loss of skeletal muscle regenerative potential. Specifically, we have found in a model of androgen deprivation, mouse castration, disruptions in Pax7 expressing skeletal muscle stem cell (satellite cell, SC) quiescence. Disruptions in SC quiescence were accompanied by reductions in Pax7+ SC numbers during skeletal muscle regeneration, and

lower levels of Spry1, an RTK/FGF feedback regulator with an essential role in the maintenance of skeletal muscle progenitors and SCs during development and regeneration^{20,21}. The present work also reveals data from transgenic mice where Spry1 is specifically overexpressed in SCs (Spry1OX mice). Preliminary data from Spry1OX mice demonstrate that SC-specific Spry1 overexpression is sufficient to attenuate declines in SC number in regenerating androgen deprived skeletal muscles. For the next reporting period we intend to use funds from this award to define the mechanisms of Pax7+ SC loss in androgen deprived skeletal muscles, and the phenotypes of regenerating androgen deprived Spry1OX mice. The latter should provide crucial insight as to whether rescue of Pax7+ SC loss is sufficient to promote androgen deprived skeletal muscle regeneration.

We intend to perform immunofluorescence analysis with various SC fate markers for proliferation, myogenic differentiation, and apoptosis in regenerating skeletal muscles of castrated mice. To assess intrinsic functional capacity we will perform clonal growth assays of SCs purified from skeletal muscles of castrated mice in defined conditions⁷. We intend to examine whether Spry1 overexpression prevents SC loss by promoting proliferation, preventing apoptosis and/or differentiation in an androgen deprived environment. In parallel we will also examine how SC specific Spry1 over expression affects regenerated skeletal muscle morphology and size.

4. Impact:

Development of the principal discipline(s) of the project:

1. Demonstrating androgen deprivation by castration leads to disruptions in Pax7 expressing skeletal muscle stem cell quiescence, a reversible state of growth arrest critical to the maintenance of stem cell function, at homeostasis.
2. Demonstrating androgen deprivation by castration leads reductions in the number of Pax7 expressing skeletal muscle stem cells during skeletal muscle regeneration.
3. Demonstrating androgen deprivation by castration leads to reductions in the size of regenerated myofibers.
4. Demonstrating androgen deprivation by castration leads to reductions in the expression of Spry1, a critical regulator of stem cell fate, in Pax7 expressing skeletal muscle stem cells.
5. Establishment of a mouse model to specifically overexpress Spry1 in Pax7 expressing skeletal muscle stem cells during regeneration in an androgen deprived environment.
6. Demonstrating specific overexpression of Spry1 is sufficient to attenuate loss of Pax7 expressing skeletal muscle stem cells during regeneration in an androgen deprived environment.

Other disciplines:

Nothing to report.

Technology transfer:

Nothing to report.

Society beyond science and technology:

Nothing to report.

5. Changes/Problems:

Approach and reasons for change:

Nothing to report.

Actual or anticipated problems or delays and action plans to resolve them:

Nothing to report.

Significant impact on expenditures:

Needed to breed more mice to obtain sufficient numbers of male Spry1OX mice of correct genotype. This created more unexpected costs.

Use or care of human subjects:

Nothing to report.

Use or care of vertebrate animals:

Needed to breed more mice to obtain sufficient numbers of male Spry1OX mice of correct genotype. This created more unexpected costs.

Use of biohazards and/or select agents:

Nothing to report.

6. Products:

Nothing to report.

7. Participants and other collaborating organizations:

Individuals that have worked on project:

Joe V. Chakkalakal (PI, no change)

Alanna Klose (no change)

Change in the active support of the PD/PI(s) or senior/key personnel:

Nothing to report.

Other organizations involved as partners:

Nothing to report.

8. Special reporting requirements:

Nothing to report.

9. Appendices:

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